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(54) Title: ENHANCER-INCREASED GENE EXPRESSION IN PLANTS

(57) Abstract

The invention provides enhancers for one or more gene promoters, which enhancers are nucleotide sequences rich in A and T bases, the total amount of A and T bases comprising more than 50 % of the nucleotide sequence. Particular sequences are identified from the pea plastocyanin promoter which are active as enhancers, as is a solely A/T nucleotide sequence, and methods of carrying out the invention are described.

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ENHANCER-INCREASED GENE EXPRESSION IN PLANTS

This invention relates to increasing the expression of a gene, and in particular, to altering the activity of the promoter of the gene.

Genetic manipulation depends on the introduction of chimaeric genes into plants and the expression of the introduced gene depends on the promoter. There are many reasons why it would be advantageous to have a method of improving the effectiveness of the promoter in these genes. Different promoters work with different efficiencies in different tissues. Different promoters work with different efficiencies in the same tissue, some, such as the Cauliflower Mosaic Virus 35S promoter (35S CaMV), are commonly considered to be a stronger promoter than the promoter from the *nos* gene. Promoters commonly consist of more that 1000bp and when shortened work less efficiently. However, long sections of DNA produce technical difficulties in recombinant DNA techniques. Therefore, there are many instances when improved expression may be required. In experiments involving antisense, the highest expression possible might be required to achieve a commercial result. It is, therefore, an advantage to have DNA constructs available which would enhance, under the appropriate conditions, the expression of a given gene.

Sequences which activate transcription have been termed enhancers (Simpson *et al.* Nature (1986) **323**, 551-554) and a sequence that is active as an enhancer has been obtained from the 35S promoter of CaMV (U.S. Patent No. 5,164,316). The 35S promoter which contains this enhancer region is active in many

plants and the promoter has been described as constitutive, acting in many tissues. However, while enhancer regions have been suggested for plant genes it has not been previously recognised that part of a plant promoter might have an enhancer activity in several different organs and in different species. For example, the -352 to -2 region of the pea *RbcS* gene was attached to the bacterial *nos* promoter and this gave strong light-induced expression in photosynthetic tissues. Similar experiments placing the element downstream of the coding sequence did not cause expression in tobacco (Fluhr *et al*, Science (1986) 232, 1106-1111).

The pea *PetE* gene was isolated by Last, D.I. and Gray, J.C. [Plant Molecular Biology (1989) **12**, 655-666]. This gene encodes plastocyanin which is a 10kDa copper protein involved in photosynthetic electron transfer. Thus, expression of this gene is required in organs such as leaves and stems in cells which contain chloroplasts. Deletion studies with the promoter region of this gene suggested that the promoter was active in leaves, stems and flowers, but not in roots, and that an element upstream from -784 to -992 repressed expression in leaves. Removal of this region produced a very 'strong' promoter (Pwee, K-H. and Gray, J.C. The Plant Journal (1993) **3** 437-449).

The invention is based on the surprising finding that a gene expressed in green photosynthetic tissue of pea has an enhancer region that is active in other species and in other tissues, including non-photosynthetic tissues.

It is an object of the present invention to provide a sequence of DNA which is active as an enhancer and causes an

increase in expression of a promoter expressed in green tissues.

It is a further object of the present invention to provide a sequence of DNA which is active as an enhancer and causes an increase in expression of any promoter which is expressed in one or more of the roots, tubers, stems, leaves, flowers or seeds of plants.

It is also an object of the invention to provide a method of enhancing expression of genes in plants other than the plant from which the sequence was obtained.

The present invention provides a method of increasing the expression of a gene promoter, the method providing an increased expression of one or more genes in one or more organs of a plant by enhancing the activity of a promoter of the one or more genes using an enhancer, the enhancer being a nucleotide sequence rich in A and T bases, the total amount of A and T bases comprising more than 50% of the nucleotide sequence.

As used herein an increase in expression means that the expression of the gene when used with the enhancer of the invention is greater than would be seen without using the enhancer of the invention in expression of that gene.

Advantageously the enhancer is either obtained from a plant gene or is synthetically produced. The enhancer may be a homologue of the plant gene or of the synthetically produced sequence.

Advantageously the enhancer is obtained from a gene expressed in pea. More advantageously, the gene is expressed

in the green photosynthetic tissues of pea, in particular, the leaves of the pea plant.

Preferably the enhanced expression of the gene to be incorporated into one or more organs of the plant is in a plant which is different from the plant from which the enhancer was obtained. The difference may be a difference in plant type, i.e. family, or another plant of the same plant family.

The present invention provides an enhancer for a gene promoter, which enhancer is a nucleotide sequence rich in A and T bases, the total amount of A and T bases comprising more than 50% of the nucleotide sequence.

The enhancer sequence may suitably be an isolated and/or purified sequence.

Preferably the sequence comprises at least 20% A bases and at least 20% T bases. Preferably the sequence comprises at least 25%, and more preferably at least 30%, and even more preferably at least 35% of A and T bases respectively. One of the A or T bases may even be present as 40, 45% or 50%, or more, of the sequence. The sequence may comprise solely A and T bases.

Advantageously the enhancer is the isolated and/or purified sequence, SEQ. ID1, of Figure 1 of the drawings hereof or a similar sequence thereto.

More advantageously the enhancer is an isolated and/or purified sub-sequence of SEQ. ID1, or a similar sequence thereto, which sub-sequence is active as an enhancer.

Preferably the sub-sequence of the enhancer is a 31bp region of SEQ. ID1 described and known herein as SEQ. ID2, or any similar sequence thereto. The enhancer may alternatively

preferably be any one or more of the following base pair sequences: -444 to -389, -388 to -284, -283 to -179, -444 to -284, -388 to -179 or any similar sequence having the required enhancer activity.

Avantageously the enhancer is the isolated and/or purified sequence, SEQ. ID3, of Figure 8 of the drawings hereof, or a similar sequence thereto.

A similar sequence may also be known as a homologue. As used herein the term homologue means a nucleic acid which has a nucleotide sequence which is identical, or similar, to another nucleotide sequence. The similarity must be sufficient to enable the nucleotide sequence to act as an enhancer according to the invention.

More advantageously the enhancer is a purified sub-sequence of SEQ.ID 3, or a similar sequence thereto, which sub-sequence is active as an enhancer.

Preferably the gene promoter is a gene promoter in plants. Preferably the sequence or sub-sequence of the enhancer causes increased expression of the gene in green or non-green tissues of plants, and in particular in the roots, tubers, seeds, stems, flowers or leaves of such plants.

Advantageously, the enhancer increases expression of a gene in plants other than the plant from which the enhancer is obtained.

Advantageously, the enhancer may comprise a plurality of enhancers. The enhancer may suitably operate both in normal or reverse orientation. Suitably the enhancer may also be operable attached to either the promoter or terminator of the gene to be expressed.

The present invention also provides a chimaeric gene comprising an enhancer according to the invention, a gene promoter, a coding or non-coding sequence and a terminator sequence.

As used herein the term chimaeric gene means a recombinant DNA molecule containing sequences from more than one organism.

The chimaeric gene may comprise more than one of the enhancer and more than one promoter.

The enhancer may be in normal or reverse orientation when contained in the chimaeric gene.

The chimaeric gene may contain a reporter sequence or any other sequence which confers an identifiable character to a transformed plant.

The present invention, moreover, provides a transformed plant, which may have been transformed by the method of the invention, having an increased expression of one or more genes in the transformed plant by virtue of the use of one or more enhancers according to the present invention.

The transformed plant may be a dicotyledonous species, such as potato, tobacco, cotton, lettuce, melon, squash, cucumber, pea, rape, soyabean, sugar beet or sunflower, or a monocotyledonous species, such as wheat, barley, rye, rice or maize. Suitable alternative transformation systems for such crops will be known to the skilled reader and need not be elucidated here.

The present invention also provides propagules of a plant transformed using an enhancer according to the present invention.

The present invention also provides a cell which harbours a gene having increased expression as a result of the method or enhancer hereof.

In order that the invention may be easily understood and readily carried into effect, reference will now be made, by way of example, to the diagrammatic drawings hereof, in which:

Figure 1 shows the specific activity of GUS in bar chart form of SEQ. ID1 behaving as an enhancer in the leaves of transgenic tobacco plants when fused in normal and reverse orientation to a minimal *PetE* promoter, GUS reporter and *nos* terminator,

Figure 2 shows the specific activity of GUS in bar chart form of SEQ. ID2 behaving as an enhancer in the leaves of transgenic tobacco plants in single and multiple copies when fused to a minimal *PetE* promoter, GUS reporter and *nos* terminator,

Figure 3 shows the specific activity of GUS in bar chart form of SEQ. ID1 behaving as an enhancer in the roots of transgenic tobacco plants when fused in normal and reverse orientation upstream or downstream of a minimal 35S CaMV(-90) promoter, GUS reporter and *nos* terminator,

Figure 4 shows the specific activity of GUS in bar chart form of sub-sequences of SEQ. ID1 in normal orientation behaving as enhancers in the leaves of transgenic tobacco plants when fused to a minimal *PetE* promcter, GUS reporter and *nos* terminator,

Figure 5 shows the specific activity of GUS in bar chart form of SEQ. ID1 behaving as an enhancer in the micro-tubers of transgenic potato plants when fused in normal and reverse

orientation to a minimal or full length patatin promoter, GUS reporter and *nos* terminator, and

Figure 6 shows the coding sequence of the pea plastocyanin promoter (-444 to -179) sequence, known also herein as SEQ. ID1,

Figure 7 shows the coding sequence for the sub-sequence of the pea pastocyanin promoter of Figure 6, this sub-sequence being known as SEQ. ID2,

Figure 8 shows the nucleotide sequence active as an enhancer and known herein as SEQ.ID3,

Figure 9 shows the specific activity of GUS in bar chart form of SEQ.ID3 behaving as an enhancer in the leaves of transgenic tobacco plants when fused to a minimal *PetE* promoter, GUS reporter and *nos* terminator,

Figure 10 shows the construct pATC, and

Figure 11 shows the constructs pATC 21040, pATC 25040, pATC 26040, pATC 27040, pATC 28040 and pATC 29040.

Example 1

The sequence known herein as SEQ. ID1 (see Figure 6) was isolated from the leaves of pea in the manner described by Last, D.I. and Gray, J.C. [Plant Molecular Biology (1989) **12**, 655-666]. This sequence was joined either in the normal or the reverse orientation to the -175 to +4 section of the *PetE* promoter fused to a GUS reporter coding sequence and *nos* terminator as shown in Figure 1. The resulting chimaeric gene in the *Agrobacterium tumefaciens* vector pBIN19 (Jefferson, R.A. *et al.*, *EMBO J*, **6**, 3901-3907) was used to transform tobacco plants (*Nicotiana tobacum cv. Samsun*). Figure 1 shows in graphical form the results for four different constructs.

For each construct several independent transformed lines were analysed. Table 1 shows the actual values of specific activity of GUS obtained for each line. As expected, the *PetE* promoter is only expressed in the leaves but not in the roots. Nevertheless, the activity figures indicate the surprising result that the -179 to -444 upstream region of this promoter will enhance expression in either orientation, i.e. normal or reverse orientation.

The methods for the production of the plants are detailed below but, as would be recognised by one skilled in the art, other methods for the production and assay of these or other plants would be equally suitable.

Transgenic plants

Recombinant fusion constructs containing the enhancer and *PetE* promoter linked to a GUS reporter and a *nos* terminator were mobilised into *Agrobacterium tumefaciens* LBA4404 (Ooms, G; Hooykaas, P.J.J; Van Veen R.J.M; Van Beelen, P; Regensburg, T.J.G; Schilpoort R.A. (1982a) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T region. Plasmid 7, 15-29) using electroporation according to Shen, W.J. and Forde, B.J. [Nucleic acid research (1989) **17**, 8385] and the transformed *Agrobacterium* cells were used to infect tobacco leaf discs according to Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. 1985 [A simple and general method for transferring genes into plants. Science, **22,** 1229-1231]. Individual kanamycin-resistant regenerated shoots were dissected away from callusing leaf discs and rooted in media without growth

regulators. Rooted transgenic plants were maintained in tissue culture on media with 100μg ml^{-1} kanamycin and 200μg ml^{-1} carbenicillin, and subcultured every 7-8 weeks. Material used in GUS assays was harvested from young, healthy, expanded leaves (25-35mm long), fairly close to the shoot apex. Roots were washed extensively in distilled water before use.

Fluorometric GUS assay

GUS enzyme assays were performed essentially according to Jefferson *et al.* 1987, *EMBO J*, **6**, 3901-3907. Extracts were made from 10-40mg of plant tissue in 500μl of GUS lysis buffer (50mM NaP_i, pH 7.0, 10 MM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10mM 2-mercaptoethanol) and 5-50μl of extract was used in each assay containing 1mM 4-methyl umbelliferyl glucuronide. Fluorescence was measured using an LS50 fluorescence spectrophotometer (Perkin Elmer, Connecticut, USA). Protein was determined using the Bradford (1976) microassay procedure.

Table 1

Specific activity of GUS from leaf and root extracts of tissue-cultured tobacco plants transformed with construct containing SEQ. ID1 in both orientations upstream of *PetE* minimal -175/+4 promoter, GUS reporter, *nos* terminator.

	Specific Activity of GUS (pmole MU/min/μg protein)	
Construct 40 *PetE* **-175/+4 promoter, GUS reporter,** *nos* **terminator as a control**		
Plant No.	**Leaves**	**Roots**
14	4.6	-
18	3.3	-
6	5.3	-
11	4.6	-
15	6.1	-
21	2.4	-
Mean±SEM	4.30±0.54	
Construct 38 SEQ.ID1 in normal orientation upstream of *PetE* **-175/+4 promoter, GUS reporter,** *nos* **terminator**		
Plant No.	**Leaves**	**Roots**
3	147.5	-
4	21.3	-
6	57.4	-
7	56.9	-
8	41.7	-
11	37.1	-
14	79.7	-
19	26.9	-
25	34.5	-
31	97.2	-
Mean±SEM	60.0±12.26	

Table 1 (continued)

Construct 39 SEQ.ID1 in reverse orientation upstream of *PetE* -175/+4 promoter, GUS reporter, *nos* terminator Plant No.	Specific Activity of GUS (pmole MU/min/µg protein) Leaves	Roots
2	60.6	-
5	43.8	-
8	55.4	-
14	160.5	-
16	24.2	-
18	85.2	-
23	78.7	-
24	16.7	-
Mean±SEM	65.6±15.96	

MU = 4 methyl umbelliferone

Example 2

In a further experiment, instead of using the whole of SEQ. ID1, a 31bp region from -289 to -259 of SEQ. ID1 was used. The sequence was modified to give SEQ. ID2 described in Figure 7.

In construct 110, one copy of the sequence SEQ. ID2 was attached to the upstream of *PetE* -175/+4 promoter, which promoter is attached to a GUS reporter coding sequence and *nos* terminator as shown in Figure 2. In construct 108, 3 copies of SEQ. ID2 were attached to the -175 to +4 *PetE* promoter. The results obtained after transformation and regeneration of tobacco plants are shown for the population in Figure 2 and indicate that more than one copy of the SEQ. ID2 region increases the expression of the promoter. Table 2 shows the actual values of specific activity of GUS obtained for each line. The same control as in Example 1 was used and as shown in Figure 2.

TABLE 2

Specific activity of GUS from leaf extracts of transgenic tobacco plants transformed with constructs containing a single copy of 31 bp fragment or 3 copies of the 31 bp fragment from the pea plastocyanin promoter that shows high affinity for protein binding.

	Specific Activity of GUS (pmole MU/min/μg protein)
Construct 110 31 bp oligonucleotide in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* promoter	
Plant No.	**Leaves**
1	5.61
2	13.99
3	4.41
4	3.20
5	3.35
6	25.30
7	10.85
8	7.35
9	4.76
10	4.58
Mean±SEM	8.34±6.54
Construct 108 3 copies of 31 bp oligonucleotide in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator	
Plant No.	**Leaves**
1	18.29
2	40.24
3	14.80
4	23.67
5	14.73
6	12.12
7	28.46
8	12.77
9	21.83
10	10.88
11	19.42
12	54.13
Mean±SEM	22.61±12.37

Example 3

To establish whether the enhancer region SEQ. ID1 had a similar effect on other promoters SEQ. ID1 was joined to the "minimal" 35S CaMV (-90) promoter in normal and reverse orientation, and also joined in normal and reverse orientation to the terminator region of the chimaeric gene prepared with the minimal CaMV promot Tobacco was transformed and assayed as described in Example 1. Figure 3 shows the mean values for the populations obtained with these constructs. Table 3 presents the values for each line both for leaves (shown in Figure 3) and for roots. The region identified as an enhancer for the *PetE* gene acts as an enhancer for the heterologous CaMV promoter and is active in both orientations, whether it is present upstream or downstream of the promoter. The enhancer is active with a promoter which is expressed in roots and leaves and, therefore, is not tissue specific.

TABLE 3

Specific activities of GUS from root and leaf extracts of transgenic tobacco plants transformed with constructs containing minimal 35S CaMV (-90) promoter, GUS reporter, *nos* terminator and the enhancer sequence.

	Specific Activity of GUS (pmole MU/min/μg protein)	
Construct 33 35S CaMV (-90) promoter, GUS reporter, and *nos* terminator as a control		
Plant No.	**Roots**	**Leaves**
4	0.07	0.30
39	0.89	0.42
48	3.21	0.35
50	0.95	0.27
51	0.98	0.39
52	1.99	0.37
53	3.44	0.33
67	5.90	0.21
77	1.25	0.47
86	2.30	0.34
Mean±SEM	2.09±0.54	0.34±0.02
Construct 34 SEQ.ID1 in normal orientation upstream of 35S CaMV (-90) promoter, GUS reporter and *nos* terminator		
Plant No.	**Roots**	**Leaves**
8	2.08	0.61
11	11.34	0.51
17	13.45	0.45
19	12.67	0.32
21	14.69	0.43
22	23.11	0.60
55	13.45	0.43
72	13.75	0.47
86	4.91	0.54
Mean±SEM	12.16±1.99	0.48±0.03

Table 3 (continued)

Construct 35 SEQ.ID1 in reverse orientation upstream of 35S CaMV (-90) promoter, GUS reporter and *nos* terminator Plant No.	Specific Activity of GUS (pmole MU/min/μg protein) Roots	Leaves
7	17.11	1.40
14	16.70	1.11
22	14.24	0.91
36	13.25	0.59
41	18.20	1.00
68	16.90	1.11
77	7.23	1.24
78	37.61	0.75
83	34.62	0.96
85	14.87	1.05
94	7.93	1.12
Mean±SEM	18.06±2.90	1.01±0.06

Construct 36 SEQ.ID1 in normal orientation downstream of 35S CaMV (-90) promoter, GUS reporter and *nos* terminator Plant No.	Roots	Leaves
5	3.34	0.36
11	2.46	0.63
14	1.49	0.51
16	1.73	0.31
21	3.28	0.26
34	7.61	0.29
36	4.29	0.38
43	2.61	0.37
49	7.67	0.36
Mean±SEM	3.83±0.77	0.38±0.03

Table 3 (continued)

Construct 37 SEQ.ID1 in reverse orientation downstream of 35S CaMV (-90) promoter, GUS reporter and *nos* terminator	Specific Activity of GUS (pmole MU/min/μg protein)	
Plant No.	Roots	Leaves
4	4.67	0.37
9	3.67	0.29
13	4.32	0.32
17	2.19	0.61
20	2.98	0.18
21	9.34	0.27
46	2.25	0.42
48	3.45	0.35
49	3.55	0.33
73	3.98	0.37
Mean±SEM	4.04±0.64	0.35±0.03

Example 4

To identify whether regions of the SEQ. ID1 other than SEQ. ID2 contained enhancer-like activity, five further chimaeric genes were constructed as depicted in Figure 4. These constructs used regions of SEQ. ID1 both upstream and downstream of SEQ. ID2. Transgenic tobacco plants were obtained as described in Example 1. Analysis of the transgenic plants containing these constructs shows that all the sub-regions of SEQ. ID1 selected contain enhancer-like activity and Table 4 indicates that the activity demonstrated by SEQ. ID1 is not wholly caused by SEQ. ID2.

Table 4

Specific activities of GUS in leaf extracts of transgenic tobaccco plants transformed with constructs containing fragments of SEQ. ID1.

	Specific Activity of GUS (pmole MU/min/μg protein)
Construct 74 -444 to -389 fragment from the pea plastocyanin promoter in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator	
Plant No.	**Leaves**
1	56.89
2	9.91
3	87.11
4	11.38
5	13.96
6	20.79
7	17.24
8	12.76
9	38.98
10	43.00
Mean±SEM	31.40±8.00
Construct 83 -388 to -284 fragment from the pea plastocyanin promoter in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator	
Plant No.	**Leaves**
1	45.55
2	16.71
3	22.32
4	21.66
5	22.49
6	17.72
7	16.86
8	11.33
9	10.81
Mean±SEM	20.60±3.43

Table 4 (continued)

	Specific Activity of GUS (pmole MU/min/μg protein)
Construct 90 -283 to -179 fragment from the pea plastocyanin promoter in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator	
Plant No.	**Leaves**
1	2.19
2	16.70
3	11.65
4	15.61
5	8.95
6	17.77
7	32.41
Mean±SEM	15.04±3.53
Construct 105 -444 to -284 fragment from the pea plastocyanin promoter in normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator	
Plant No.	**Leaves**
1	42.81
2	30.29
3	28.63
4	31.82
5	24.68
6	34.67
7	33.54
8	22.95
9	25.61
10	44.81
11	37.89
Mean±SEM	32.51±2.15

Table 4 (continued)

Construct 111 -388 to -179 fragment from the pea plastocyanin promoter in the normal orientation upstream of *PetE* -175/+4 promoter, GUS reporter and *nos* terminator Plant No.	Specific Activity of GUS (pmole MU/min/μg protein) Leaves
1	11.43
2	36.72
3	51.10
4	21.45
5	32.22
6	17.61
7	8.75
Mean±SEM	25.61±5.73

Example 5

To establish whether the enhancer region was active in other species the -179 to -444 region was joined to the -330 to +1 region of the patatin promoter PS20 described by Mignery, G.A.; Pikaard, C.S. and Park, W.D. 1988 [Molecular characterisation of the patatin multigene family of potato. Gene, **62**, 27-44]. The chimaeric gene produced was transferred into potato by transformation.

Plant Material

Potato shoot cultures were maintained *in vitro* on Murashige and Skoog (MS) medium in Magenta GA-7 containers at 22°C (16 hours/8 hours light/dark). These were nodally sub-cultured every 3 weeks.

In vitro shoots of 2-3 inches (5-7.5cm) height were potted in 2.5 inches (6.4cm) pots of Levingtons F1 compost. They were weaned in a propagator for one week in a growth room at 18°C

(16 hours/8 hours light/dark). The propagator was removed and the plants repotted at 3 weeks into 5 inch (12.7cm) pots. At 5-7 weeks the plants were used for transformation.

Agrobacterium tumefaciens

Liquid overnight cultures of suitable strains, e.g. LBA4404, C58#3, were grown at 28°C to an OD_{600} (Pharmacia LKB ULTRASPEC II) of 0.8 in L-broth (see below).

Cocultivation

The youngest four most expanded leaves were taken and surface sterilised in 10% commercial bleach (Domestos RTM) for 15 minutes. Leaves were rinsed thoroughly with sterile water and then cut into discs with a 7mm cork borer. The discs were mixed with the *Agrobacterium* for 1-5 minutes, blotted dry on filter paper (Whatman No. 1) and then placed on callusing medium (see below) in 90mm triple vented petri dishes, lower epidermis down. The 90mm triple vented petri dishes were sealed with tape, cut to allow gas exchange and then incubated at 22°C (16 hours/8 hours light/dark). The discs were transferred to callusing medium plus 500μg ml^{-1} of claforan and 30μg ml^{-1} kanamycin after 48 hours. This removes bacteria and selects for transformed cells.

Regeneration of Transformed Shoots

After 1 week, the discs were transferred to shooting medium containing the same antibiotics.

L-broth	10g l^{-1} bactotryptone
	5g l^{-1} yeast extract
	5g l^{-1} sodium chloride
	1g l^{-1} glucose
Callusing medium	MS with 3% sucrose
	0.5mg l^{-1} 2,4-D
	2.5mg l^{-1} BAP
Shooting medium	MS with 3% sucrose
	2.5mg l^{-1} BAP
	1.0mg l^{-1} GA_3

Further transfers were made onto the same medium until shoots could be excised (usually about 4 weeks). Shoots with calli were transferred to MS medium with claforan (500μg/ml) in well ventilated containers, e.g. Magenta. Transformants were maintained, after several passages with cefotaxime to remove bacteria, on MS medium. They were removed from *in vitro*, weaned and grown to maturity as described above for plant material. The process yields transformed potato plants at a frequency of up to 30% of the discs cocultivated.

Microtubers produced in the presence of Ancymidol (180μg/ml) from these plants were assayed for GUS activity as described in Example 1. Figure 5 shows the results and indicates that the enhancer sequence in either orientation can

increase the activity of the patatin promoter and Table 5 shows that the gene was expressed in micro-tubers but not in leaves.

TABLE 5

Specific activity of GUS from micro-tubers induced on transgenic potato plants containing SEQ. ID1 upstream of a -330 to +1 patatin class 1 promoter compared with a minimal patatin promoter and a full length PS20 patatin promoter.

	Specific GUS activity (pmole MU/min/μg protein)	
Construct p250 2562 bp patatin promoter, GUS reporter and *nos* terminator		
Plant No.	**Micro-tubers**	**Leaves**
1	51.38	
2	6.17	
3	32.50	
4	49.28	
5	15.96	
6	10.36	
Mean±SEM	27.55±8.06	
Construct 116 330/+1 bp minimal patatin promoter, GUS reporter and *nos* terminator		
Plant No.	**Micro-tubers**	**Leaves**
1	3.85	
2	14.01	
3	10.15	
4	8.45	
5	9.03	
6	8.79	
7	8.81	
Mean±SEM	9.01±1.12	

Table 5 (continued)

	Specific Activity of GUS (pmole MU/min/μg protein)	
Construct 112 -444 to -179 fragment from the pea plastocyanin promoter in normal orientation upstream of minimal patatin -330/+1 promoter, GUS reporter and *nos* terminator		
Plant No.	**Micro-tubers**	**Leaves**
1	18.76	
2	15.28	
3	8.79	
4	14.66	
5	11.29	
6	84.08	
7	16.28	
8	61.15	
9	12.77	
10	10.14	
11	9.49	
Mean±SEM	23.88±7.48	
Construct 114 -444 to -179 fragment of the pea plastocyanin promoter in reverse orientation upstream of minimal patatin -330/+1 promoter, GUS reporter and *nos* terminator		
Plant No.	**Micro-tubers**	**Leaves**
1	18.36	
2	40.82	
3	40.49	
4	11.81	
5	98.54	
6	59.89	
7	11.48	
8	15.26	
9	7.50	
Mean±SEM	33.69±10.01	

Example 6

The sequence known herein as SEQ.ID3 (see Figure 8) was designed by coin flipping and constructed from two complementary oligonucleotides (5' AAT TAT AAT ATA ATT TTA ATT

TAA AA3') and (5' AAT TTT TTA AAT TAA AAT TAT ATT AT 3') containing *Eco*RI overhangs at the 5' ends to allow multimerisation without any intervening G/C bp. Oligonucleotides were annealed, phosphorylated and concatamers were inserted in the *Eco*RI site of pIC19H (Marsh *et al*, 1984). Sequencing identified three plasmids containing inserts of 4, 2 and 1 copies of the oligonucleotide, respectively. Inserts were excised as *HindIII-SalI* fragments and inserted in pKHd7 (Pwee, K-H and Gray, J.C. (1993) The Plant Journal), known herein as pJSS22, to give pJSS139, pJSS140 and pJSS141 containing 2,1 and 4 copies respectively.

The resulting chimaeric gene in the *Agrobacterium tumefaciens* vector pBIN19 was used to transform tobacco plants (*Nicotiana tobacum cv. Samsum*) according to the methods described above. Figure 9 shows in graphical form the results for five different constructs. For each construct several independent transformed lines were analysed. Table 6 shows the actual values of specific activity of GUS obtained for each line. As expected, the *PetE* promoter is expressed in the leaves but also indicate the surprising result that multiples of SEQ.ID3 will enhance expression in a dose related manner.

Table 6

Specific GUS activity from leaf extracts of transgenic tobacco plants transformed with a construct containing single copy of 26 mer oligonucleotide at *Eco*RI site upstream of *petE* minimal -175/+4 promoter, GUS reporter and *nos* terminator.

Specific GUS activity (pmol MU/min/μg protein)

Plant No.	No. 26 mer	1-26 mer	2-26 mer	3-26 mer
1	4.6	6.14	20.73	18.87
2	3.3	4.01	12.60	52.40
3	5.3	5.83	6.39	27.38
4	4.6	2.68	2.56	7.59
6	2.4	2.21	42.95	
7			7.57	
8			5.57	
9			10.08	
10			4.82	
Mean±SEM	**4.30±0.54**	**3.91±0.69**	**16.46±5.39**	**35.68±11.70**

Example 7

To further establish that SEQ ID1 was active with other promoters the pea metallothionein promoter, known herein as $PsMT_A$, (Marta Evans, I., *et al.*, FEBS **262** (1) 29-32) was obtained. The region -806 to -1 of that promoter was ligated to the GUS coding region which contained an intron (Vancanneyt, G. *et al.*, (1990) Mol. Gen. Genet **220** 245-250) and a *nos* terminator, which resulted in construct pKS 21040 (see Figure 10). SEQ ID 1 was joined at the 5' end to this chimaeric gene in either the normal or reverse orientation. The enhancer-promoter-GUS-*nos* terminator fusions were moved into the binary vector pATC (a pBIN19 derivative with modified restriction sites), shown in Figure 10. This resulted in the constructs pATC 25040 and pATC 26040 respectively (see Figure 11) which were used to transform *Nicotiana tabacum* cv Heavy Western tobacco with *Agrobacterium tumefaciens* R1000 to produce hairy roots.

Hairy root transformation:

Agrobacterium tumefaciens R1000 (McAfee, B et al., Plant Cell Tissue and Organ Culture (1993) **34**, 53-62) was transformed by electroporation (Shen, W.J. and Forde, B.J. Nucleic acid research (1989) **17**, 8385) and the resulting bacteria used to transform Nicotiana tabacum by the leaf disc method (Horsch, R.B; Fry, E.J; Hoffman, N.L; Eichholtz, D; Rogers, S.G; Fraley, R.T. Science (1985) **22**, 1229-1231). Roots which were resistant to kanamycin were dissected away from discs and transferred to

tissue culture media which contained 100μg/ml kanamycin and 500μg/ml claforan. Roots were maintained in this media and subcultured every three weeks. The roots were then transferred to media with 100μg/ml kanamycin and 200μg/ml claforan. After a further 7 days the roots were transferred to media without the claforan and subcultured every week.

Example 8

Monomer, dimer and tetramers of the synthetic oligomer (SEQ. ID 3) were synthesised to contain *EcoRI* overhangs so that they could be ligated into the *Eco*RI site of pIC19H (Marsh *et al* (1984) Gene 32, 481-485). The *XhoI- SalI* fragment was the excised from the resulting plasmids and ligated into the corresponding site in pKS 21040. The synthetic oligomer promoter-GUS-nos terminator fusions were moved into the binary vector pATC. The resulting constructs pATC27040, pATC28040 and pATC29040 (see Figure 11) were used to transform *Nicotiana tabacum* cv Heavy Western to produce hairy roots.

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CLAIMS

1. An enhancer for a gene promoter, which enhancer is a nucleotide sequence rich in A and T bases, the total amount of A and T bases comprising more than 50% of the nucleotide sequence.
2. An enhancer according to Claim 1, wherein the enhancer is an isolated and/or purified sequence.
3. An enhancer according to Claim 1 or 2, wherein said nucleotide sequence comprises at least 20% A bases and at least 20% T bases.
4. An enhancer according to Claim 3, wherein the sequence comprises at least 25% of A and T bases respectively.
5. An enhancer according to Claim 4, wherein the sequence comprises at least 30% of A and T bases respectively.
6. An enhancer according to Claim 5, wherein the sequence comprises least 35% of A and T bases respectively.
7. An enhancer according to any one of Claims 1 to 6, wherein one of the A or T bases is present as 40, 45% or 50%, or more, of the sequence.
8. An enhancer according to any one of Claims 1 to 7, wherein the sequence may comprise solely A and T bases.
9. An enhancer according to Claim 1, wherein the enhancer is the isolated and/or purified sequence, SEQ. ID1, of Figure 1 of the drawings hereof or a similar sequence thereto.
10. An enhancer according to Claim 9, wherein the enhancer is an isolated and/or purified sub-sequence of SEQ. ID1, or a similar sequence thereto, which sub-sequence is active as an enhancer.

11. An enhancer according to Claim 10, wherein the sub-sequence of the enhancer is a 31bp region of SEQ. ID1 described and known herein as SEQ. ID2.

12. An enhancer accirding to Claim 10, wherein the enhancer is alternatively any one or more of the following base pair sequences: -444 to -389, -388 to -284, -283 to -179, -444 to -284, -388 to -179, or any similar sequence having the required enhancer activity.

13. An enhancer according to Claim 1, wherein the enhancer is the isolated and/or purified sequence, SEQ. ID3, of Figure 8 of the drawings hereof, or a similar sequence thereto.

14. An enhancer according to Claim 13, wherein the enhancer is a purified sub-sequence of SEQ.ID 3, or a similar sequence thereto, which sub-sequence is active as an enhancer.

15. A method of increasing the expression of a gene promoter, the method providing an increased expression of one or more genes in one or more organs of a plant by enhancing the activity of a promoter of the one or more genes using an enhancer, the enhancer being a nucleotide sequence rich in A and T bases, the total amount of A and T bases comprising more than 50% of the nucleotide sequence.

16. A method of increasing the expression of a gene promoter according to Claim 15, wherein the enhancer is obtained from a plant gene or is synthetically produced.

17. A method of increasing the expression of a gene promoter according to Claim 16, wherein the enhancer is a homologue of the plant gene or the synthetically produced sequence.

18. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 17, wherein the gene promoter is a gene promoter in plants.

19. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 18, wherein the sequence or sub-sequence of the enhancer causes increased expression of the gene in green or non-green tissues of plants, and in particular in the roots, tubers, seeds, stems, flowers or leaves of such plants.

20. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 19, wherein the enhancer comprises a plurality of enhancers.

21. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 20, wherein the enhancer operates both in normal or reverse orientation.

22. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 21, wherein the enhancer is attached to either the promoter or terminator of the gene to be expressed.

23. A method of increasing the expression of a gene promoter according to any one of Claims 15 to 23, wherein the enhancer is any one of Claims 1 to 14.

24. A chimaeric gene comprising an enhancer according to any one of Claims 1 to 14, a gene promoter, a coding or non-coding sequence and a terminator sequence.

25. A chimaeric gene according to Claim 24, said chimaeric gene comprising more than one of said enhancer and more than one promoter.

26. A plant having an increased expression of one or more genes ins by virtue of the use of one or more enhancers according to any one of Claims 1 to 14.

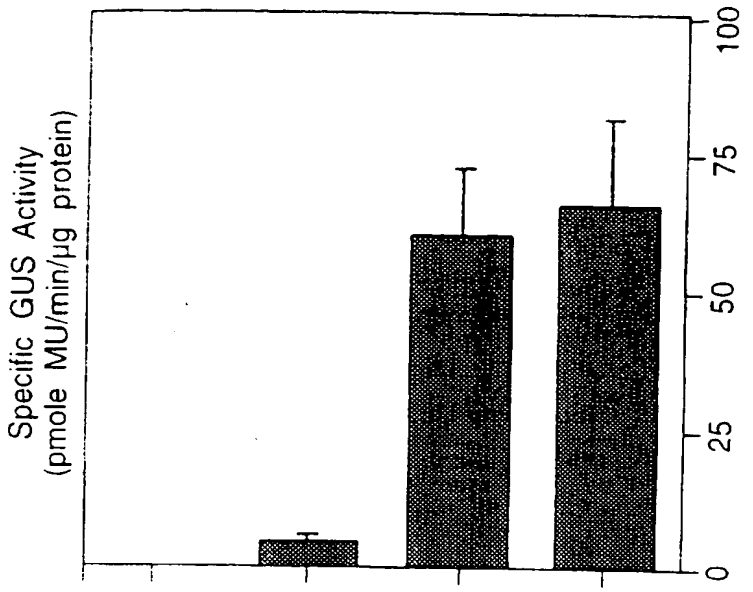
27. A plant according to Claim 26, wherein the transformed plant may be a dicotyledonous species, such as potato, tobacco, cotton, lettuce, melon, squash, cucumber, pea, rape, soyabean, sugar beet or sunflower, or a monocotyledonous species, such as wheat, barley, rye, rice or maize.

28. Propagules of a plant transformed using an enhancer according to any one of Claims 1 to 14.

29. A cell which harbours a gene having increased expression as a result of the method of any one of Claims 15 to 23 or the enhancer of any one of Claims 1 to 14 hereof.

30. A chimaeric gene substantially as hereinabove described with reference to any one of Figures 1 to 5 and 11 hereof.

31. A nucleotide sequence substantially as hereinabove described with reference to any one of Figures 6, 7 or 8 hereof in use as an enhancer.



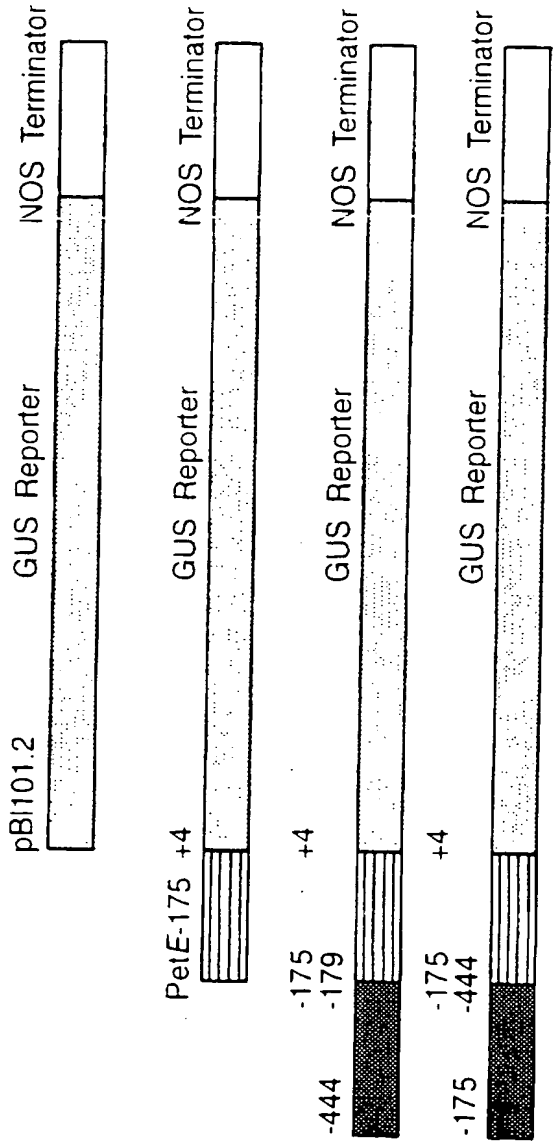


Fig.1.

Fig.2.

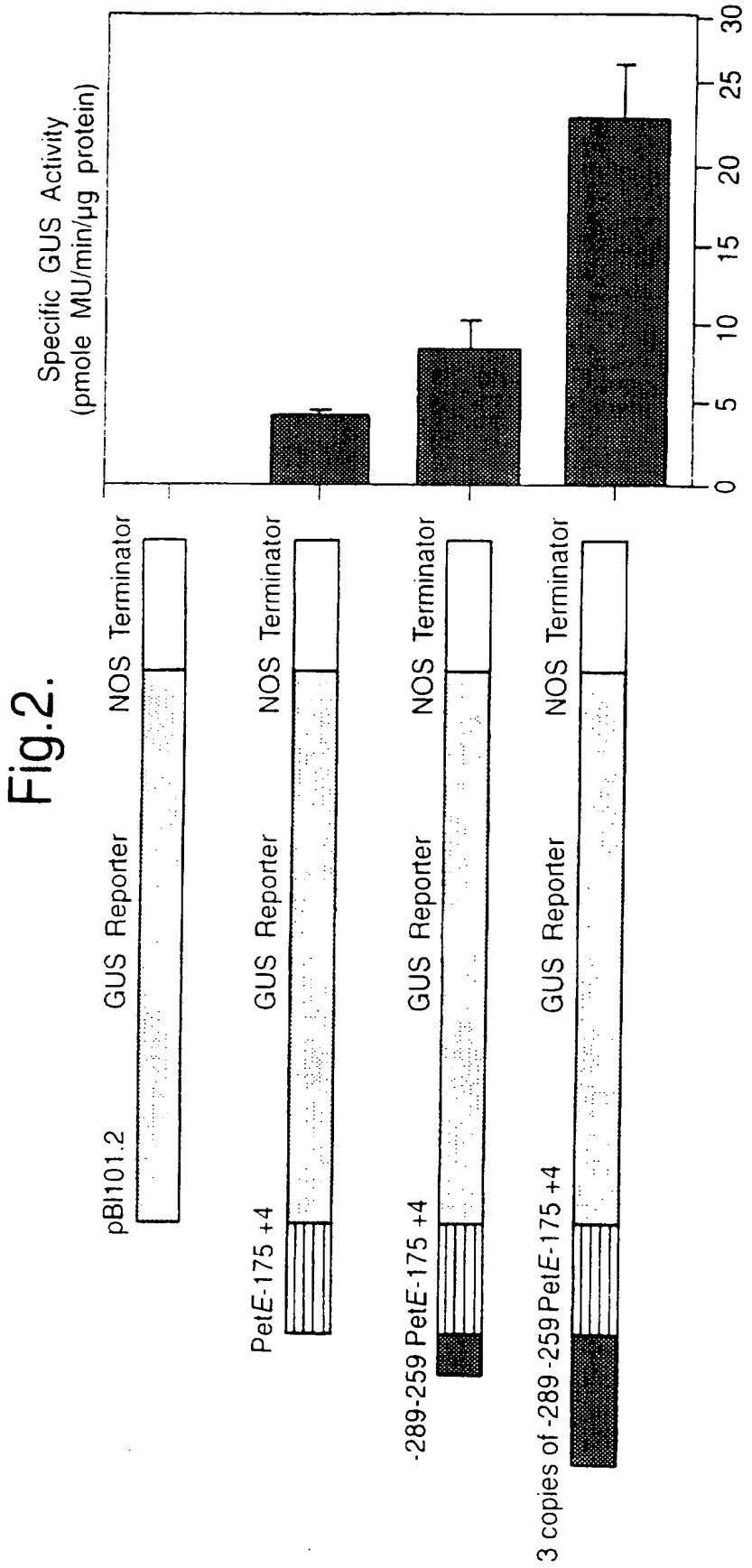


Fig.3.

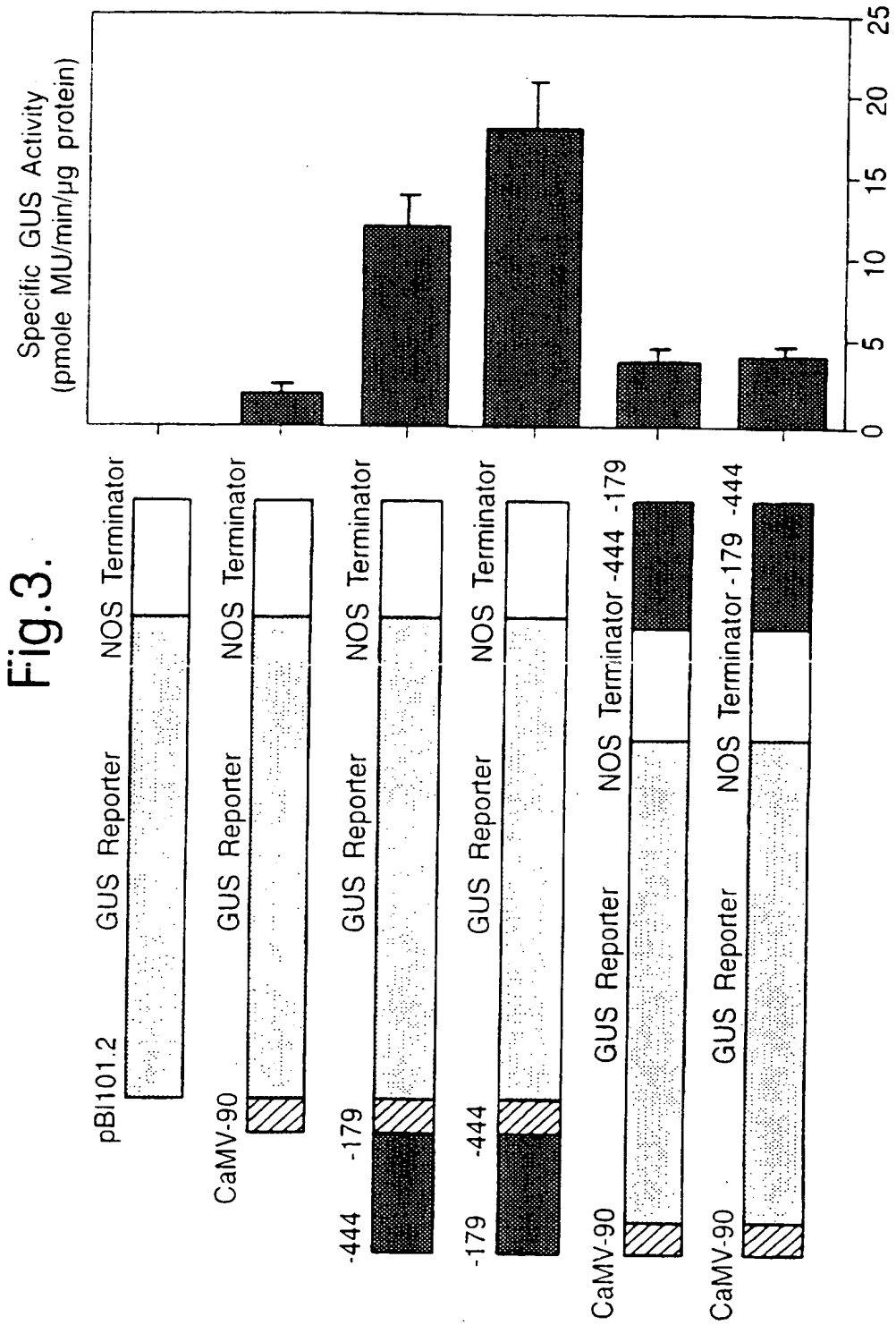


Fig.4.

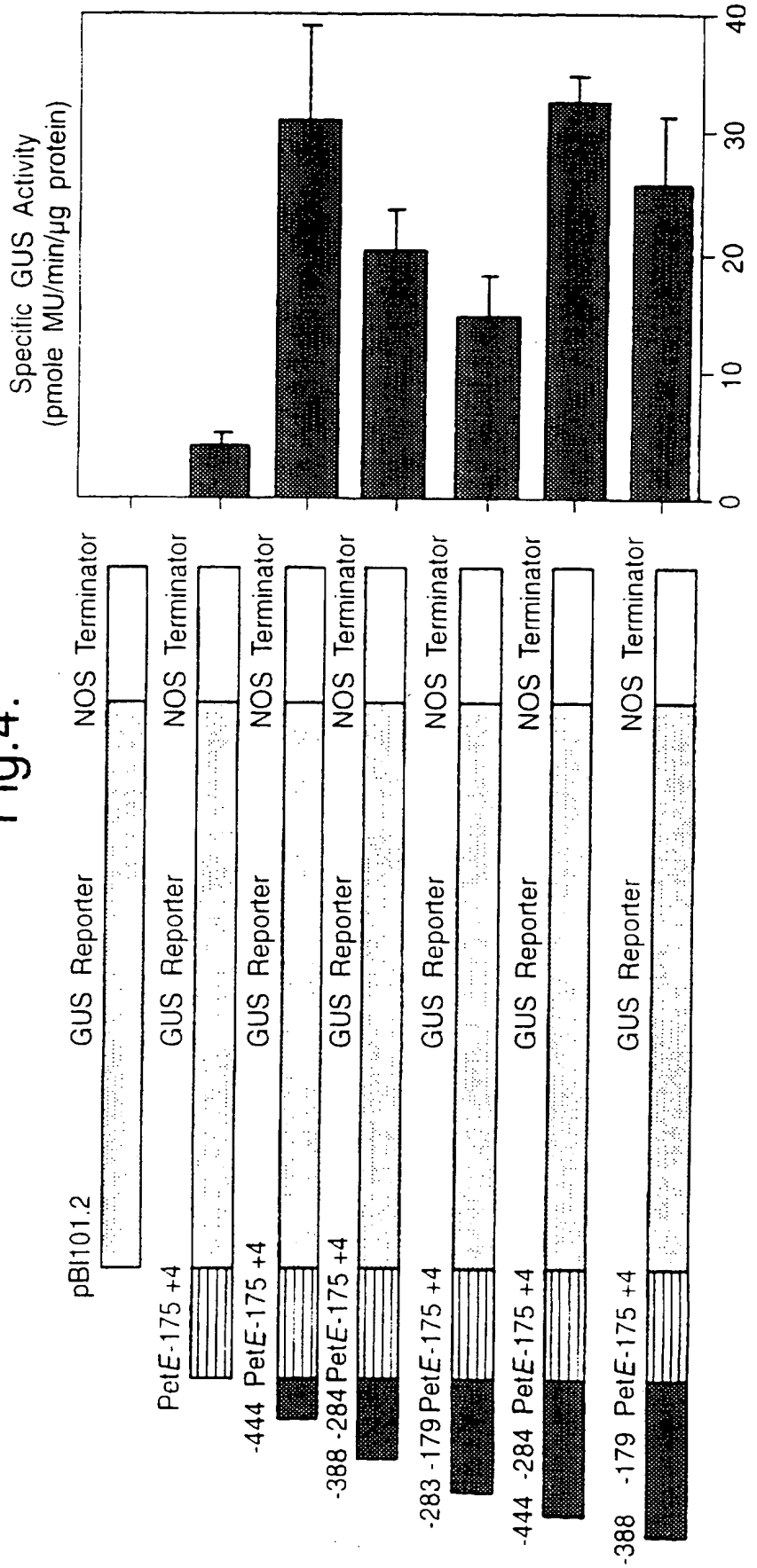
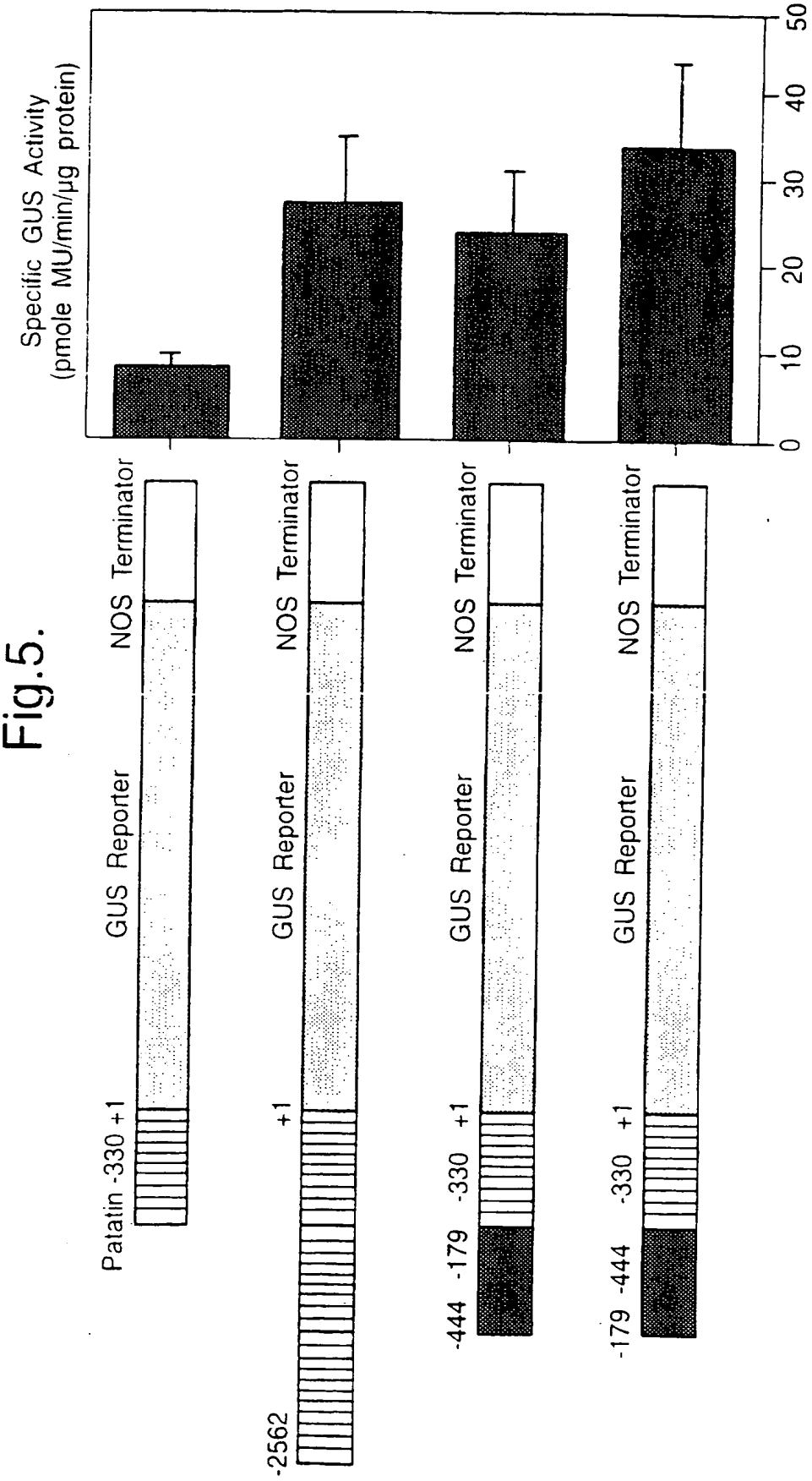


Fig.5.



SEQ. ID1

Fig.6.

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-444 AGCTTAGTTA ATCATGTTAA ACAACAATTC TTTGTAATAA TAAAATTGTC 50
-394 TTTCAACTAG TCCAAGTTTA TGAGTTGATT CTTCGGAATA AATTAGAAAA 100
-344 TATCTTAGAT TTTATACTTC ATTGATTATT TCATAGAGCA AGTAGGAGAA 150
-294 ATAAAAATAT ACTAGTATTA TTTACTAAAA AAAATCTAAG CCACGTCGGA 200
-244 GGATAACATC CAACCCAGCC AATCACAGCA ATGTTCATCA GATAACCCAC 250
-194 TTTAAGCCCA CGCACT                                    266
```

Length: 266
Type: Genomic
Strandedness: Single
Topology: Linear

Fig.7.

SEQ. ID2

GATC AATATACTAG TATTATTTAC TAAAAAAAAT C
TTATATGATC ATAATAAATG ATTTTTTTTA G CTAG

Length: 31
Type: Genomic
Strandedness: Double
Topology: Linear

Fig.8.

SEQ. ID3

AATT ATAATATAAT TTTAATTTAA AA
TATTATATTA AAATTAAATT TT TTAA

Length: 26
Type: Synthetic
Strandedness: Double
Topology: Linear

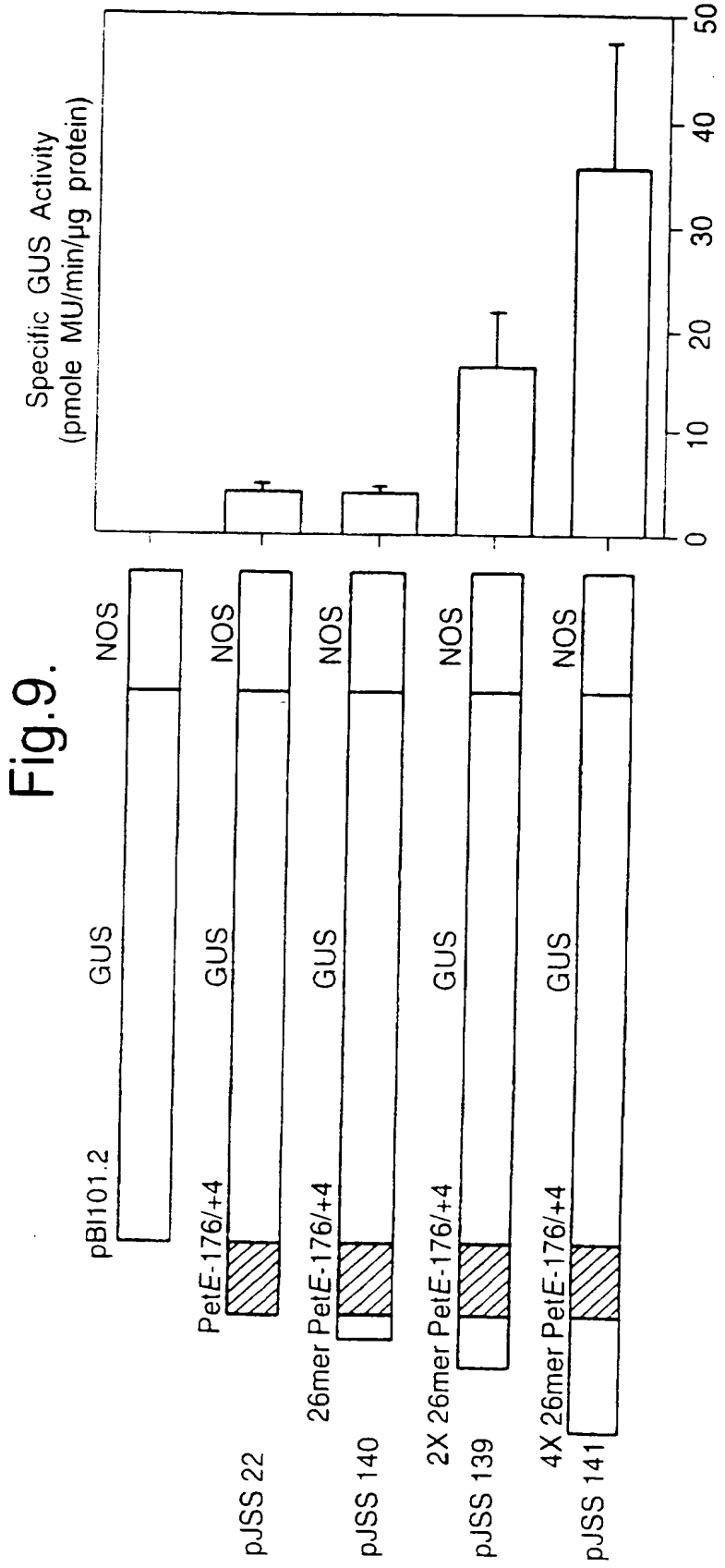

Fig.9.

Fig.10.

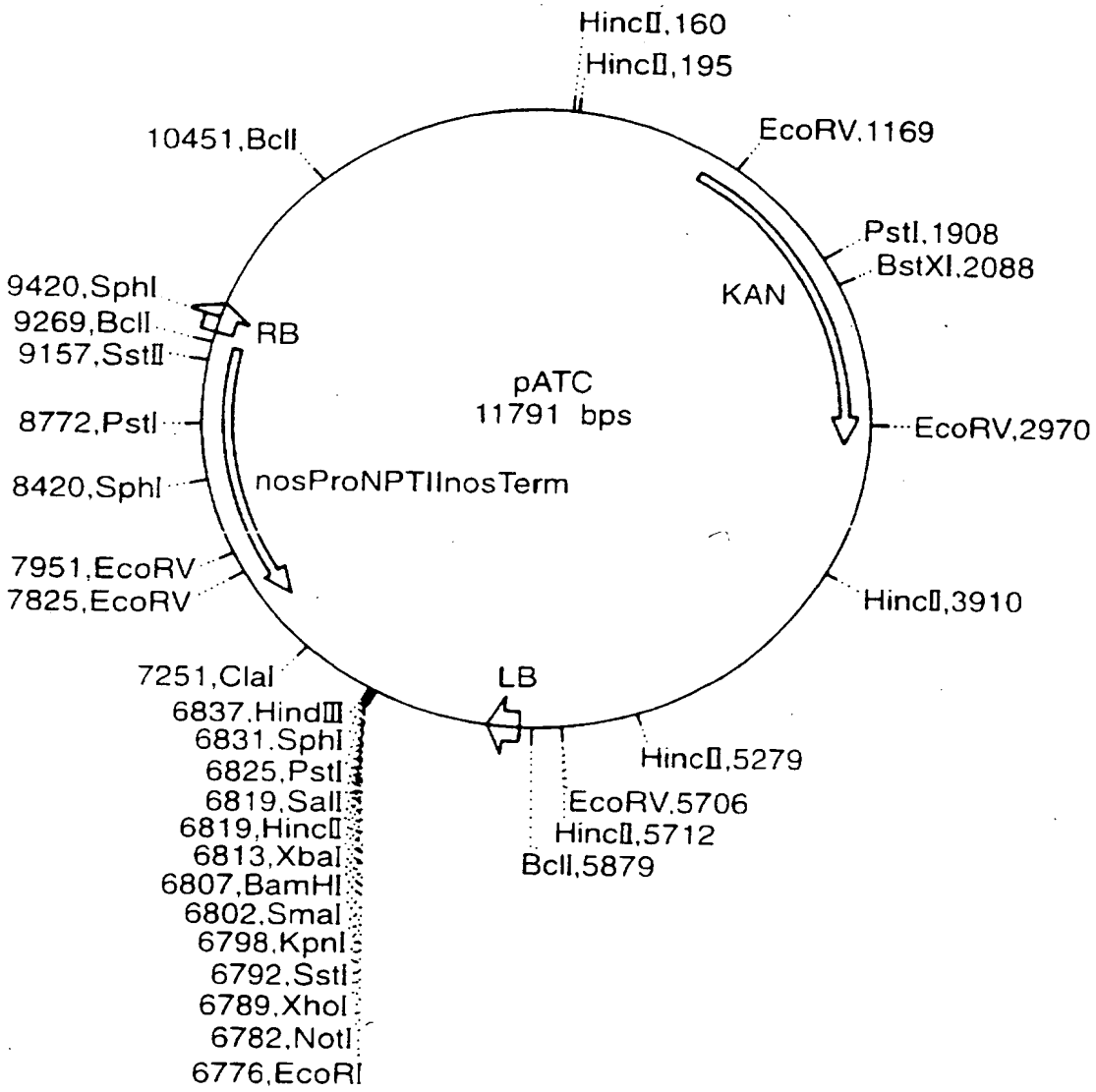

Fig.11.